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Immunolocalization of antimicrobial and cytoskeletal components in the serous glands of human sinonasal mucosa

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Summary. Secretory cells in the seromucous glands of paranasal sinuses secrete antibacterial proteins for innate immune mucosal integrity. We studied the localization of antimicrobial and cytoskeletal components of the human seromucous glands and respiratory epithelium of the maxillary sinus and the ethmoidal cells by immunohistochemical methods. The presence of a variety of defense proteins such as lysozyme, lactoferrin, cathelicidin, and defensin-1, -2, -3 point to a crucial role in the immune defense for the respiratory tract. Cytoskeletal proteins such as actin, myosin 2, cytokeratin 7 and 19, α- and β-tubulin, investigated for the first time in glands of paranasal sinuses, showed a stronger expression at the apical and lateral cell membrane. The localization of the cytoskeletal proteins might point to their participation in exocrine secretory processes and stabilizing effects.

Key words: Human sinonasal mucosa, Serous glands, Antimicrobial Proteins, Cytoskeleton, Immunohistochemistry

Introduction

Paranasal sinuses are air-filled spaces communicating with the nasal cavity. Both paranasal and nasal cavities are lined by respiratory epithelium with numerous goblet cells. In addition, seromucous glands are found in the respiratory mucosa of the paranasal sinuses (Ovalle and Nahirney, 2008). As the glands of the nasal mucosa, they are composed of mixed tubuloalveolus endpieces containing mucous and serous cells or solely mucous or serous endpieces. No intercalary- or striated ducts are found. Intralobular ducts join interlobular ducts merging and forming the main duct (Terrahe, 1970).

The nose and sinuses as a part of the upper respiratory tract play a crucial role as a protective system against inhaled pathogens, such as Gram-positive and Gram-negative bacteria, fungi, and enveloped viruses. Cationic antimicrobial peptides are known for their broad-spectrum antimicrobial activity against these microorganisms (Daher et al., 1986; Wu et al., 2005; Woodworth et al., 2006; da Silva et al., 2012). There are several studies investigating the expression of proteins of the innate immune system of the nasal mucosa (Chen and Fang, 2004; Ooi et al., 2007) or nasal secretions (Cole et al., 1999; Niehaus et al., 2000). Detailed histological investigations of antimicrobial proteins and peptides in the glands of the sinus mucosa are rare. ß-Defensins are cationic peptides acting against a broad range of bacteria (Wu et al., 2005; Yamaguchi and Ouchi, 2012) and were found in epithelial cells, e.g. keratinocytes (Harder et al., 2004), secretory glands (Stoeckelhuber et al., 2006, 2008; Kesting et al., 2012a), lung (Scharf et al., 2012), and at the apical side of the pseudostratified ciliated epithelial cell cytoplasm of maxillary sinus mucosa (Carothers et al., 2001). ß-Defensin mRNA was expressed in inferior turbinate mucosa (Lee et al., 2002). Cathelicidin plays a critical role in the human innate immune defense against

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bacterial infection (Zanetti, 2005; Stoeckelhuber et al., 2008) and was detected in the epithelium and submucosal glands of the nasal mucosa (Kim et al., 2003; Ooi et al., 2007). In the respiratory system, the two predominantly found antimicrobial proteins are lactoferrin and lysozyme (Bowes et al., 1981; Dubin et al., 2004; Woods et al., 2011). Serous cells of submucosal mixed glands in maxillary sinus mucosa displayed a positive staining reaction for lysozyme and lactoferrin in the normal mucosa (Fukami et al., 1993).

Cytoskeletal proteins are important for the regulation of a number of functions in the cell including cell motility (dos Remedios et al., 2003), endocytosis (Qualmann and Kessels, 2002), and exocytosis (Stoeckelhuber et al., 2012). The interaction of actin with non-muscle myosin 2 clustered around secretory vesicles supports exocytosis in lacrimal acinar cells (Jerdeva et al., 2005) and plays a role in the exocytosis of serous glandular cells in the human submandibular gland (Stoeckelhuber et al., 2012). Cytokeratins were detected in the decapitation region of apocrine glandular cells in the human ceruminous glands (Stoeckelhuber et al., 2006), in the human glands of Moll (Stoeckelhuber et al., 2003), and in the human axillary apocrine glands (Stoeckelhuber et al., 2011). Thus, cytokeratins are part of a contractile machinery at the cell surface leading to the aposome pinching-off. Also, the coexistent eccrine secretion mode in these glandular cells depends on the presence of cytokeratins (Takuma et al., 1993). In milk secreting cells, microtubules may play a role in transport of secretory vesicles to the apical regions for exocytosis (Nickerson and Keenan, 1979). This study provides the first insights into the localization of cytoskeletal proteins in the serous glands of sinonasal mucosa

Materials and methods

Tissue and patients

Tissue of human sinus mucosa was obtained from 47 patients (30 males and 17 females; age range, 15-79 years) undergoing surgery for different indications. Healthy, non-infected tissue was used. 40 samples were excised from the maxillary sinus, 7 from the ethmoidal cells. The study was performed according to the guidelines of the Ethics Comitee. The material was fixed immediately in buffered formalin (4.5%) following dehydration and embedding in paraffin wax.

HE and immunohistochemical staining

Five micron paraffin sections were stained with hematoxylin and eosin (HE) for histological orientation, with periodic acid Schiff (PAS) reagent for neutral carbohydrates and with Alcian-blue for polyanions according to Romeis (2010).

Immunohistochemical staining was performed with antibodies to lysozyme (Thermo Scientific, Rockford, USA), lactoferrrin (Abcam, Cambridge, UK), cathelicidin (Thermo Scientific, Rockford, USA), ß-defensin-1 (Santa Cruz Biotechnology, Santa Cruz, USA), ß-defensin-2 (Santa Cruz Biotechnology, Santa Cruz, USA), ß-defensin-3 (Novus Biologicals, Littleton, USA), actin (Abcam, Cambridge, USA), myosin 2 (Sigma, St. Louis, USA), cytokeratin 7 (Dako, Hamburg, Germany), cytokeratin 19 (Dako, Hamburg, Germany), α-tubulin (Novus Biologicals, Cambridge, UK), ß-tubulin (Santa Cruz Biotechnology, Santa Cruz, USA). The primary antibodies were applied according to the avidin-biotin-horseradish-peroxidase complex using the Vectastain Kit from Vector (Vector Laboratories, Inc.) and diluted as follows: anti-lysozyme 1:2000, anti-lactoferrin 1:100, anti-cathelicidin 1:50, anti-ß-defensin-1, -2, -3 1:100, anti-actin 1:50, anti-myosin 2 1:1000, anti-cytokeratin 7 1:100, anti-cytokeratin 19 1:50, anti-α-tubulin 1:200, anti-ß-tubulin 1:200. The first antibody was applied to the tissue for 1h at room temperature and overnight at 4°C. All sections were pretreated with microwave irradiation in citrate buffer at pH 6.0 for 15 min and with 3% hydrogen peroxide for 10 min. Blocking of non-specific binding was performed by incubation with swine serum, goat serum or BSA (bovine serum albumin) for 30 min. The second, biotinylated antibody (Vector Laboratories Inc., Burlingame, USA) was diluted 1:200 and applied for 45 min at room temperature. Sections were then incubated with peroxidase-labelled streptavidin (Vector Laboratories Inc., Burlingame, USA) for 45 min at room temperature and peroxidase activity was visualized by diaminobenzidine. Counterstaining was done with hematoxylin. Negative controls, in which the primary antibody was replaced by buffer, were treated identically. Mouse or rabbit IgG, as appropriate, at the same concentration as the first antibody was used as isotype control for each protein. In Table 1 the staining procedures for each antibody are summarized. Sections were viewed with a Nikon Eclipse 80i microscope, and images were taken with a digital Nikon camera (Nikon, Düsseldorf, Germany). Labeling intensities were categorized as weak, medium, and strong staining.

Table 1. Primary antibodies.

antibody	host	clone	dilution	blocking serum
Anti-lysozyme	rabbit	polyclonal	1:2,000	swine (3%)
Anti-lactoferrin	mouse	monoclonal	1:100	BSA (2%)
Anti-cathelicidin	rabbit	polyclonal	1:50	swine (3%)
Anti-ß-defensin-1	rabbit	polyclonal	1:100	goat (3%)
Anti-ß-defensin-2	rabbit	polyclonal	1:100	goat (3%)
Anti-ß-defensin-3	rabbit	polyclonal	1:100	goat (3%)
Anti-actin	mouse	ACTN05 (C4)	1:50	goat (3%)
Anti-myosin 2	rabbit	polyclonal	1:1,000	goat (3%)
Anti-CK7	mouse	OV-TL 12/30	1:100	swine (3%)
Anti-CK19	mouse	RCK108	1:50	swine (3%)
Anti-α-tubulin	mouse	monoclonal	1:200	swine (3%)
Anti-ß-tubulin	mouse	monoclonal	1:200	swine (3%)

Results

General morphology

Groups of cross-sectioned serous and mucous glands were located in the connective tissue underneath the respiratory epithelium of the maxillary sinus mucosa (Fig. 1a-c). Flat serous cells, so-called serous demilunes, were found at the ends of mucous glandular cells. The serous exocrine part of the gland consisted of serous acini surrounding a lumen that was strikingly often wider than in typical serous glands (Fig. 1d). Intralobular ducts were lined with simple cuboidal epithelium (Fig. 1d) becoming multilayered in larger intra- and extralobular ducts. The glandular epithelium showed basically located myoepithelial cells. In some sections, groups of serous glands without mucous parts were visible in the connective tissue. We could not find differences in structure between the glands of the sinus mucosa and the ethmoidal cells.

Lysozyme

The lysozyme staining pattern showed strong staining of the serous glandular cells. Often, the protein was expressed in distinct granules in the cytoplasm (Fig. 2a). In some glandular cells, the apical part of the cell was more strongly stained. The respiratory epithelium remained unstained, occasionally displaying faint positive staining (Fig. 3a).

Lactoferrin

The cytoplasm gave medium to strong staining with

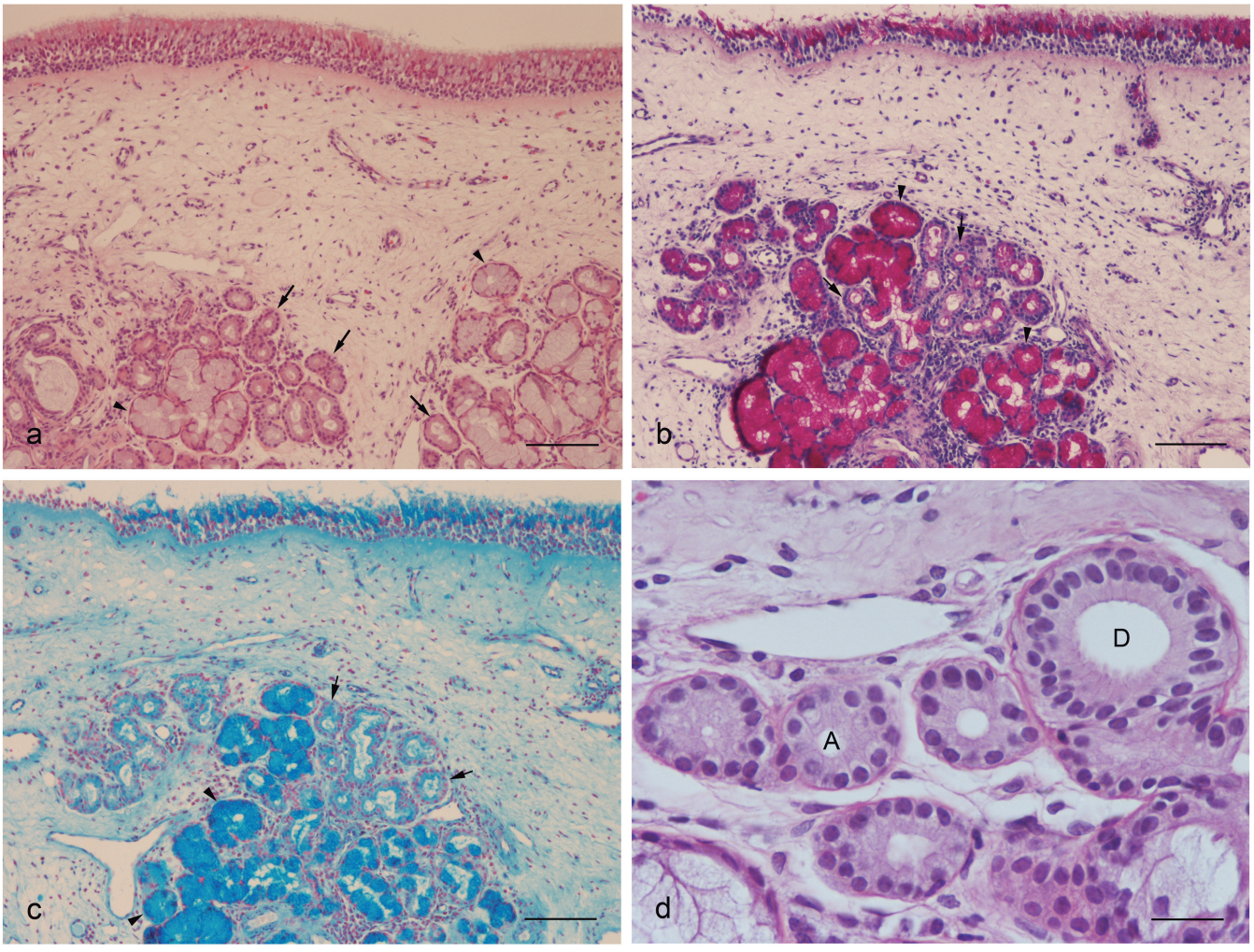

Fig. 1. Light micrographs of serous (arrows) and mucous (arrowheads) glands of the maxillary sinus mucosa. **a.** HE stain. **b.** PAS stain. **c.** Alcian-blue stain. **d.** Serous acini (A) and intralobular duct (D) of sinus mucosa glands. HE stain. Scale bar: a-c, 100 μm; d, 25 μm.

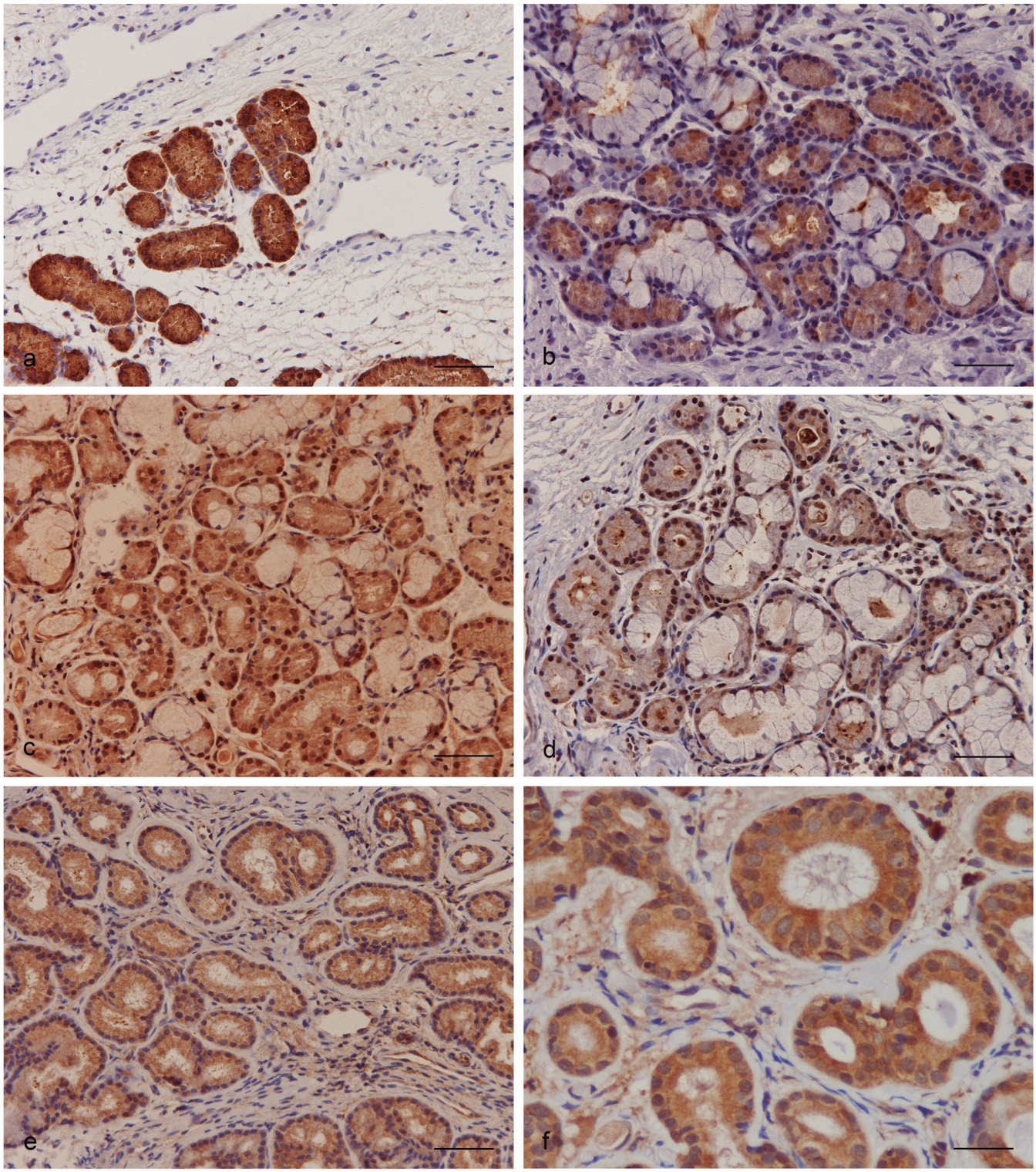

Fig. 2. a. Anti-lysozyme immunoreactivity in a granular staining pattern. **b.** Strong lactoferrin staining in serous glandular cells. **c.** Anti-cathelicidin antibody stained the whole cytoplasm with medium to strong intensity. Antibody to ß-defensin-1 reacted with medium staining reaction **(d)**, while ß-defensin-2 **(e)** and ß-defensin-3 **(f)** showed variable staining patterns. Scale bar: a-e, 50 μm; f, 25 μm.

an apical stronger marking (Fig. 2b). In some cases a more or less granular staining could be observed. In most cases, the mucosa epithelium showed no staining with the antibody to lactoferrin. In some cases a very weak staining pattern was found (Fig. 3b).

Cathelicidin

Cathelicidin was expressed with medium to strong intensity in the whole cytoplasm of the serous glandular cells (Fig. 2c) and also in the respiratory epithelium (Fig. 3c).

Defensin-1, 2, 3

A medium reaction was observed with the antibody to defensin-1 in the serous glandular cytoplasm (Fig. 2d). Defensin-2 showed a variable staining pattern with weak, medium (Fig. 2e) and occasionally strong staining. Defensin-3 reacted positively in the acini of the glandular cells (Fig. 2f). The staining intensity varied from medium to strong. Antibodies to defensin-1 and -2 reacted in the mucosa epithelium with medium to strong expression (Figs. 3d, e), while defensin-3 immunoreactivity was predominantly strong (Fig. 3f).

Actin

Medium to strong staining-intensity was detected in serous glandular cells with the anti-actin antibody (Fig. 4a). The apical and the lateral cell membrane were more strongly stained.

Myosin 2

Myosin 2 was expressed with medium to strong intensity (Fig. 4b). Partially, stronger staining at the luminal and basolateral lumen could be observed.

Cytokeratin 7 and 19

Cytokeratin 7 (CK7) and cytokeratin 19 (CK19) exhibited medium to strong staining pattern in serous acini in all sections (Figs. 4c/d). The apical and lateral cell membrane stained more strongly.

α- and β-tubulin

Antibodies to α- and β-tubulin showed the same staining results. Weak to medium staining of the cytoplasm could be observed. Partially, stronger apical staining of the serous glandular cells was seen (Figs. 4e/f).

Controls

Both negative controls and isotype incubation with mouse or rabbit IgG for each protein (Fig. 5) showed no staining.

Discussion

We investigated the serous glands and respiratory epithelium of the mucosa of the maxillary sinus and the ethmoidal cells with regard to the expression of antimicrobial proteins. In addition, components of the cytoskeleton in the serous glands were analysed. ß-Defensin-1 mRNA was studied in inferior turbinate mucosa of healthy tissue and of material from patients with chronic sinusitits and could be detected in all samples (Lee et al., 2002). These findings support our results showing medium staining intensity. However, no immunohistochemical staining method was performed in this study, thus, the definite localization of ß-defensin-1,

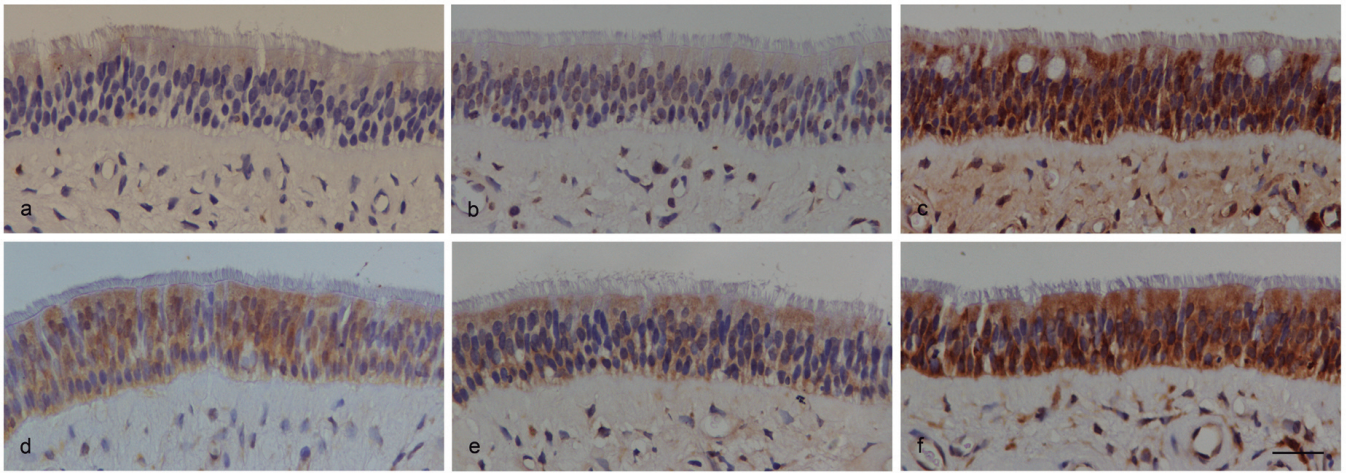

Fig. 3. Respiratory epithelium stained with antibodies to lysozyme **(a)**, lactoferrin **(b)**, cathelicidin **(c)**, defensin-1 **(d)**, defensin-2 **(e)** and defensin-3 **(f)**. Scale bar: 25 μm.

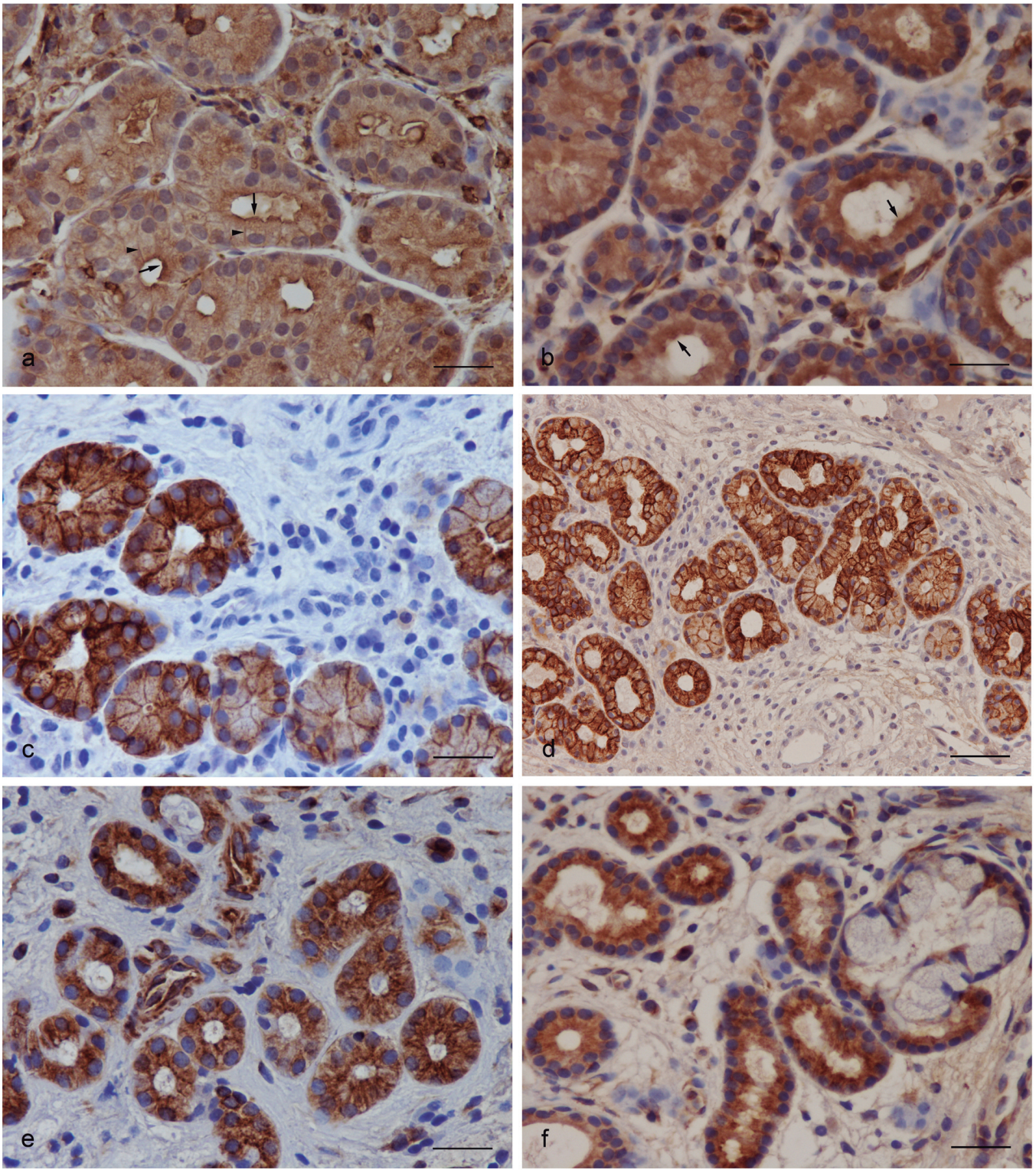

Fig. 4. a. Actin was highly expressed in the apical (arrows) and lateral cellular (arrowheads) membrane. **b.** Antibody to myosin 2 exhibited strong apical staining (arrows). CK7 **(c)** and CK19 **(d)** immunoreactivity was intensified in the apico-lateral cellmembrane. α- **(e)** and β-tubulin **(f)** was localized in the whole cytoplasm, occasionally apically more strongly stained. Scale bar: a-c, e, f, 25 μm; d, 50 μm.

whether it is in the epithelium or glands or other free cells was not described. ß-Defensin-2 mRNA was not detected in normal turbinate tissues (Lee et al., 2002) but was found in turbinate samples obtained from patients with chronic sinusitis. Although, ß-defensin-2 expression is inducible at the transcriptional level by bacterial LPS (McNamara et al., 1999) or stimulated by interleukin 1-ß (McDermott et al., 2003), it is also produced constitutively, e.g., in the human lung (Bals et al., 1998), in the human ceruminous glands (Stoeckelhuber et al., 2006), and in the serous endpieces of the sinus mucosa glands, as described in this study.

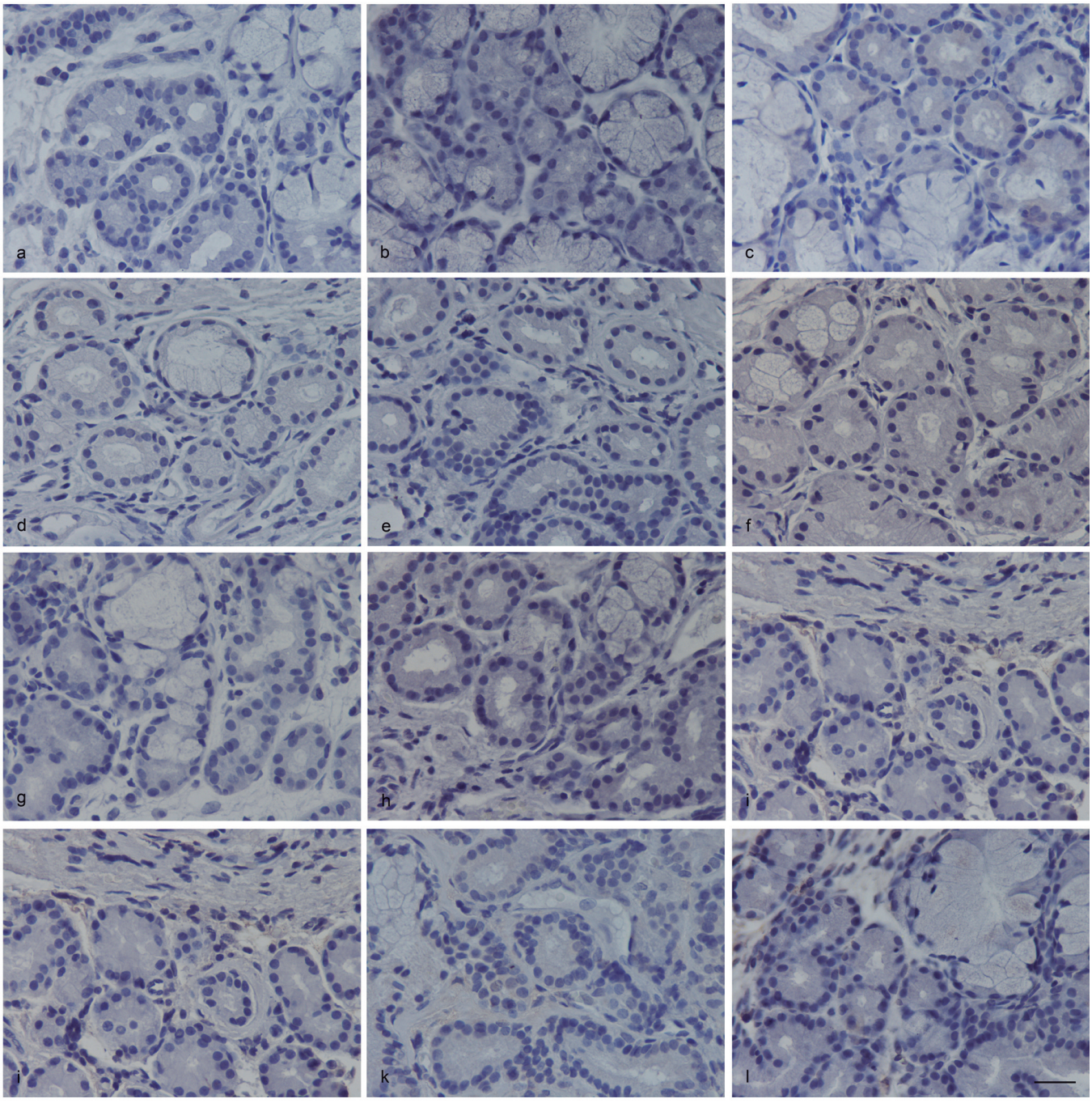

Fig. 5. Isotype control antibodies: lysozyme **(a)**, lactoferrin **(b)**, cathelicidin **(c)**, ß-defensin-1 **(d)**, ß-defensin-2 **(e)**, ß-defensin-3 **(f)**, actin **(g)**, myosin 2 **(h)**, CK 7 **(i)**, CK19 **(j)**, α-tubulin **(k)**, ß-tubulin **(l)**. Scale bar: 25 μm.

Carothers et al. (2001) localized ß-defensin-1 and -2 to the apical side of the pseudostratified ciliated epithelial cell cytoplasm of normal maxillary sinus mucosa; glandular structures were not investigated. ß-defensin-3 is present at the transcriptional level in the oral mucosa and extraoral skin (Kesting et al., 2012b), within the perichondrium and cartilage matrix layers of the nasal and auricular cartilage (Warnke et al., 2010). ß-Defensin-3 was also constitutively expressed in the glands of sinus mucosa in our study. Positive staining of cathelicidin could be found in normal nasal mucosa tissues, especially in the cytoplasm of surface epithelial cells and the submucosal glands (Chen and Fang, 2004). In chronic nasal inflammatory disease, cathelicidin is upregulated (Kim et al., 2003). Our results for submucosal glands in human maxillary and ethmoidal sinus were in accordance with the findings of the nasal mucosa tissue. Usually, epithelial cells contain both lactoferrin and lysozyme; however, with a different contribution (Brandtzaeg, 1984). Fukami et al. (1993) described a medium to strong staining intensity of lysozyme and lactoferrin in normal submucosal glands in human maxillary sinus. We found the same immunoreactivity pattern of these two antimicrobial proteins. While immunohistochemical studies of lactoferrin reported an upregulation in inflammatory tissue (Fukami et al., 1993), at mRNA and protein level a decrease could be observed in patients with chronic rhinosinusitis (Psaltis et al., 2007). Woods et al. (2012) found a low concentration of lysozyme-like immunoreactivity in some sub-epithelial glands of normal sinus mucosa.

This is the first study to examine cytoskeletal components in serous glandular cells of the sinus mucosa. Stronger actin-immunoreactivity of the apical and lateral glandular cell membrane could be observed. Increased turnover of apical actin filaments was detected in lacrimal acinar epithelial cells (Jerdeva et al., 2005). F-actin separates the secretory granules from the luminal membrane in parotid and submandibular glands (Segawa et al., 1998). The F-actin remodelling process is supposed to move vesicles to the cell surface membrane for membrane fusion (Lang et al., 2000). Myosin 2 is a molecular motor protein binding to actin. It plays a role in exocytosis at the luminal part of the glandular cells. This reflects the strong apical myosin 2 staining of glandular sinus mucosa cells. Myosin 2 seems to regulate the fusion pore opening (Bhat and Thorn, 2009) and contributes to the contractile force of granule content expulsion (Yu and Bement, 2007). In our investigation, the colocalization of actin and myosin 2 supports the interaction of actin with non-muscle myosin 2 in the secretory process. In endocrine or exocrine glandular cells, secretory granules are transported along microtubule systems from the basal to the apical cytoplasm (Malaisse et al., 1975; Nickerson and Keenan, 1979). Apical stronger staining of α- and ß-tubulin in serous glandular cells of the sinus mucosa was also detected in the human ceruminous gland (Stoeckelhuber et al., 2006), and the non-lactating human mammary gland (Welsch et al., 2007). Oriolo et al. (2007) described the cortical location of keratin intermediate filaments in single-layered epithelial cells which are found apically or apico-laterally. This was confirmed by our findings of the localisation of Cytokeratin 7 and Cytokeratin 19 at the apical and lateral cell membrane. Similar results were obtained for the human axillary apocrine gland (Stoeckelhuber et al., 2011), the human ceruminous gland (Stoeckelhuber et al., 2006) and the human glands of Moll (Stoeckelhuber et al., 2003). Besides the stabilizing effect, cytokeratins are important for vesicle transport. In addition, intermediate filaments are of high importance for directionality in vesicle mobility in mouse astrocytes (Kreft et al., 2009).

The human seromucous glands of the maxillary sinus and the ethmoidal cells contribute to the innate immunity of the sinus mucosa by expressing antimicrobial proteins like lysozyme, lactoferrin, cathelicidin, and defensin-1, -2, -3. In contrast, the respiratory epithelium displayed only distinct cathelicidin- and defensin- immunoreactivity, whereas lysozyme and lactoferrin were hardly expressed. These findings emphasize the importance of the serous glands as a source of the constitutive expression of anitimicrobial proteins. Immunohistochemical staining for cytoskeletal proteins like actin, myosin 2, α- and ß-tubulin, CK 7 and 19 in the apicolateral region of serous glands of paranasal sinuses might point to their involvement in exocytosis and stabilization.

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